T Cell Receptor (TCR) Structure of Autologous Melanoma-reactive Cytotoxic T Lymphocyte (CTL) Clones: Tumor-infiltrating Lymphocytes Overexpress In Vivo the TCR β Chain Sequence Used by an HLA-A2-restricted and Melanocyte-lineage-specific CTL Clone

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Summary

HLA-A2$^+$ melanomas express common melanoma-associated antigens (Ags) recognized in vitro by autologous cytotoxic T lymphocytes (CTL). However, it is not known whether tumor Ags can drive in vivo a selective accumulation/expansion of Ag-specific, tumor-infiltrating T lymphocytes (TIL). Therefore, to evaluate this possibility, 39 CTL clones isolated from several independent mixed lymphocyte tumor cultures (MLTC) of TIL and peripheral blood lymphocytes (PBL) of an HLA-A2$^+$ melanoma patient and selected for T cell receptor (TCR)–dependent, HLA-restricted tumor lysis, were used for analysis of TCR α and β chain structure by the cDNA polymerase chain reaction (PCR) technique with variable gene-specific primers followed by sequencing. Despite absence of oligoclonality in fresh TIL and PBL, as well as in T cells of day 28 MLTC (day of cloning), sequence analysis of TCR α and β chains of TIL clones revealed a dominance of a major category of melanoma-specific, HLA-A2–restricted T cells expressing a Vα8.2/JαAP511/Cα and Vβ2.1/Dβ1/Jβ1.1/Cβ1 TCR. The same TCR was also found in 2 out of 14 PBL clones. The other PBL clones employed a Vα2.1 gene segment associated with either Vβ13.2, 14, or w22. Clones A81 (Vα2.1/JαIGRJα04/Cα and Vβ14/Dβ1/Jβ1.2/Cβ1) and A21 (Vα8.2/JαAP511/Cα and Vβ2.1/Dβ1/Jβ1.1/Cβ1), representative of the two most frequent TCR of PBL and TIL, respectively, expressed different lytic patterns, but both were HLA-A2 restricted and lysed only HLA-A2$^+$ melanomas and normal melanocytes, thus indicating recognition of two distinct HLA-A2–associated and tissue-related Ags. Finally, by the inverse PCR technique, the specific TCR β chain (Vβ2.1/Dβ1/Jβ1.1/Cβ1) expressed by the dominant TIL clone was found to represent 19 and 18.4% of all Vβ2 sequences expressed in the fresh tumor sample and in the purified TIL, respectively, but <0.19% of Vβ2$^+$ sequences expressed in PBL. These results are consistent with the hypothesis that a clonal expansion/accumulation of a melanocyte-lineage–specific and HLA-A2–restricted T cell clone occurred in vivo at the site of tumor growth.

Studies on the T cell immune response to human melanoma have provided evidence that the mechanism of recognition of melanoma Ags by CTL is similar to that of more conventional types of Ags, namely recognition of complexes between tumor-associated peptides and MHC class I molecules by a cell surface heterodimer receptor (TCR) composed of an α and β chain (1–4). The first peptide–HLA complex recognized as a melanoma Ag has been recently identified

(5), but it is likely that melanomas from different patients or even from the same patient will express multiple Ags. In fact, distinct antigenic determinants, as well as different HLA class I alleles acting as restricting elements, have been defined on melanoma cells by autologous T cell clones (1–4, 6, 7). Whether or not the degree of complexity of the melanoma Ag repertoire will be reflected in a corresponding repertoire of distinct TCR recognizing those Ags is unknown, but re-

cent evidence from this laboratory is in agreement with the hypothesis that the CTL-mediated response to melanoma is polyclonal at the single patient level. This evidence comes from the observation that different CTL clones from the same patient may have similar antitumor reactivity but different TCR structure (8).

Most of the studies that have addressed the question of TCR diversity in melanoma, or in other neoplastic diseases, have analyzed fresh or IL-2-cultured tumor-infiltrating T lymphocytes (TIL)[1] in the attempt to identify oligoclonal T cell populations characterized by a restricted set of different TCR gene segments. The identification of such a population of T cells, if present, could facilitate the study of the molecular mechanisms of the immune response and could provide a specific target for new immunotherapeutic strategies. Indeed, in agreement with the possibility of identifying oligoclonal T cell populations in the lymphoid infiltrate, a preferential expression of Vα7 TCR gene segment has been found in primary human uveal melanoma by PCR using V gene family specific primers in seven out of eight patients studied (9). On the other hand, the TCR Vα/Vβ repertoire of fresh TIL from metastatic lesions of cutaneous melanoma has been found to vary widely even in the same patient (10, 11). Furthermore, after long-term culture in IL-2, TIL from metastatic melanoma may become oligoclonal in their TCR β chain rearrangements (12–14), although it is difficult to assess the mechanism of this process.

The analysis of TCR structure of whole TIL populations, however, will hardly allow one to link the information on TCR structure and repertoire with the functional involvement of those TCR in the antitumor response, since one cannot distinguish between tumor-specific and bystander lymphocytes with no antitumor reactivity. Moreover, this approach does not allow the association of any defined combination of TCR α and β chains with a precise antigenic specificity, and thus fails to provide a clear answer to the question of whether melanoma can drive, at the site of tumor growth, a preferential expansion/accumulation of tumor-specific T cells recognizing melanoma Ags.

To address these issues, we initially determined the Vα/Jα and Vβ/Dβ/Jβ TCR gene segments employed by a large set of TIL or PBL CTL clones from a HLA-A2$^+$ melanoma patient. The results indicated that different TCR combinations can confer different antitumor specificity. Moreover, a dominant HLA-A2-restricted T cell clone expressing a Vα8.2/JαAP511/Cα, Vβ2.1/Dβ1/Jβ1.1/Cβ1 TCR and directed to a melanocyte-lineage-associated Ag could be found in a majority of TIL clones independently isolated from different mixed lymphocyte tumor cultures (MLTC). By inverse PCR (IPCR), the nucleotide sequence corresponding to the specific TCR β chain of this dominant TIL clone was found to represent 18.4 and 19% of all Vβ2$^+$ sequences in the fresh tumor sample and fresh, purified TIL, respectively, compared

with <0.19% of Vβ2$^+$ sequences expressed in PBL. These results suggest that the expression on the tumor of a melanocyte–lineage–associated Ag can influence the tissue distribution of a T cell clone recognizing that Ag.

Materials and Methods

Neoplastic Cells. All melanoma cells used in this study were isolated from primary or metastatic lesions obtained from patients admitted for surgery to the Istituto Nazionale Tumori and were established in vitro as previously described (7). In particular, Me9742/1 and Me9742/2 are a LN and a subcutaneous metastatic lesion, respectively, from melanoma patient 9742 (HLA-A2, -A24; -B13, -B18; -Cw6, -Cw7; -DR7, -DR11; and -DQ4). All tumors were kept in culture with RPMI 1640 (MA Bioproducts, Walkersville, MD) supplemented with 10% FCS. Normal human melanocytes isolated from neonatal foreskin were cultured as described (15). The biological and phenotypical characterization of the melanocytes has been reported recently (15).

Lymphocyte Cultures. PBL and TIL were isolated as described (15) from peripheral blood and from one of the metastatic lesion (Me9742/2) of patient 9742. Immediately after isolation, PBL and TIL were divided into five separated aliquots, each initially cultured in 24-well plates (model 3424; Costar Corp., Cambridge, MA) at 2.5 × 10^5–10^6 cells/ml in 2 ml of RPMI 1640 supplemented with 20% pooled human serum, antibiotics, and 50 IU/ml of rIL-2 (kindly provided by EuroCetus, Amsterdam, The Netherlands). PHA (Wellcome Diagnostics, Research Triangle Park, NC) at 1 μg/ml was added to one aliquot of TIL and PBL for mitogenic stimulation (TIL-PHA, PBL-PHA). MLTC were started by adding viable tumor cells at a lymphocyte/tumor ratio of 5:1 to each of the remaining cultures (named TIL-1, -2, -3, -4 and PBL-1, -2, -3, -4). All independent MLTC, as well as mitogenic stimulations, were later transferred into 25 cm^2 flasks (model 25100B; Bibby Scientific Products Ltd., Stone, UK) and were restimulated every 7 d for 3 wk before cloning at the same conditions as above. Cloning was carried out from the four independent MLTC/TIL and the four independent MLTC/PBL cultures by limiting dilutions at 5, 1, 0.5, 0.25 cells/well in round-bottomed 96-well plates (model 3596; Costar Corp.) in the presence of 50 IU/ml rIL-2, irradiated (3,000 rad) allogeneic PBL as feeders (5 × 10^4/well), and irradiated (10,000 rad) autologous tumor cells (5 × 10^3/well). The plates were restimulated with autologous tumor and feeder cells after 1 wk of culture and screened at day 14 for the presence of growing clones. Clones were also derived by plating fresh PBL at 0.5 and 0.25 cells/well in the presence of allogeneic irradiated PBL, 50 IU/ml rIL-2, and 1 μg/ml PHA. The probability of clonality for each clone was calculated by Poisson statistics as described by Taswell et al. (16), and only clones with a p value for clonality >0.95 were further analyzed. LAK cells were obtained by culturing PBL from normal donors in 500 IU/ml rIL-2. Samples from the surgical tumor specimen, freshly isolated TIL and PBL, MLTC and PHA cultures, and T cell clones, maintained for 2 wk after the initial screening in the absence of feeder cells, were stored at −70°C and used to extract total RNA. TIL and PBL clones were also cryopreserved for future use.

Immunofluorescence Analysis. Indirect immunofluorescence was performed on autologous MLTC, PHA cultures, or T cell clones and analyzed on a FACS® (Becton Dickinson & Co., Mountain View, CA) as previously described (17). mAbs used were OKT3 (anti-CD3, CRL8001; American Type Culture Collection (ATCC), Rockville, MD), OKT4 (anti-CD4, CRL8002; ATCC), OKT8

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[1] *Abbreviations used in this paper:* IPCR, inverse PCR; MLTC, mixed lymphocyte tumor culture; TIL, tumor-infiltrating lymphocyte.

(anti-CD8, CRL8014; ATCC), and WT31 (anti-TCR-α/β, Becton Dickinson & Co.).

Cytotoxicity Assay. Cytotoxic activity of MLTC, T cell clones, and LAK cells was measured in a standard 4-h ^{51}Cr-release assay on panels of target tumors as previously described (7). Inhibition of lysis by mAb to CD3 (OKT3), HLA class I (W6/32; SeraLab, Sussex, UK), HLA-A2, (CR11.351; kindly provided by Dr. C. Russo, Cornell University, New York) (18), and HLA-DR (D1.12; kindly provided by Dr. R. Accolla, University of Verona, Italy) (19) was performed as described (7) with mAbs used at the final concentration of 1 μg/ml. The percent specific lysis was calculated using the following formula: 100 × [(Experimental release [cpm] − spontaneous release [cpm])/(maximum release [cpm] − spontaneous release [cpm])].

Results were also expressed in lytic units (LU), where one LU is defined as the number of effectors required for 30% lysis and the number of LU present in 10^7 effector cells was calculated.

RNA Extraction and cDNA Synthesis. Total RNA from the different samples was prepared by the method of Chomczynski and Sacchi (20) using RNAzol™B (Cinna/Biotecx, Friendswood, TX). Single-stranded cDNA synthesis was carried out on 2 μg of total RNA with oligo-dT and Moloney murine leukemia virus–derived reverse transcriptase without RNase H activity (MMLV RT RNase H$^-$, Superscript; Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. After an ethanol precipitation, the cDNA was suspended in 40 μl water.

PCR and IPCR. PCR was carried out by amplification of TCR-α and -β cDNA with oligonucleotide primers complementary to TCR V and C region sequences (9, 21) in 25 μl of a reaction mixture containing 0.5 μl of cDNA, 200 μM of each dNTP, 1 μM of each primer, and 0.625 U of Taq polymerase (Ampli Taq) on a DNA thermal cycler (all from Perkin Elmer Cetus Corp., Emeryville, CA). Reaction mixtures were subjected to 30 amplification cycles, each consisting of a 1-min denaturation step at 95°C, a 1-min annealing step at 55°C, and a 1-min extension step at 72°C. Expression of Vα or Vβ genes was considered positive when a rearranged band (200–500 bp) was visualized on 2% agarose gels with ethidium bromide staining. For some experiments, Southern blot analyses were performed by transferring to nylon membranes (Hybond N$^+$; Amersham International, Buckingham-shire, UK), 2% agarose gels containing a Vα-Cα or Vβ-Cβ rear-ranged band, and hybridizing the blots with internal C region oli-gonucleotides (probes A and B, respectively, see Table 1) or with oligonucleotides corresponding to the V/Jα and V/D/Jβ junctional regions (probes C and D, Table 1) of TIL clones expressing a TCR composed of Vα8.2/JαAP511/Cα and Vβ2.1/Dβ1/Jβ1.1/Cβ1. IPCR was performed according to the method described by Ue-matsu (22). Briefly, double-stranded cDNA was synthesized by stan-dard methods (23) from 1 μg of total RNA, blunt-ended with T4 DNA polymerase (New England Biolabs Inc., Beverly, MA), and circularized with T4 DNA ligase (Gibco BRL) in a volume of 10 μl. The ligated material (1 μl) was used as PCR template in a 50-μl reaction mixture. The couple of C region β chain primers used are identical to the ones described by Uematsu et al. (24) and are listed in Table 1 as oligonucleotides E (forward primer) and F (inverse primer). Oligonucleotides E and F contain artificial SalI and NotI sites, respectively, for subsequent directional cloning. At the end of the 30 amplification cycles (denaturation at 95°C for 40 s, annealing at 62°C for 40 s, and extension at 72°C for 1 min), the Klenow fragment of *Escherichia coli* DNA polymerase I (New England Biolabs Inc.) was added to ensure full-length DNA syn-thesis.

Cloning of PCR and IPCR Amplified Products. The amplified products of PCR obtained with V and C gene specific primers (9,

21) were ligated to pCR200™ vector (TA cloning kit; Invitrogen, San Diego, CA) which was subsequently used to transform *E. coli* competent cells. The PCR 5' V region primer used in conjunction with Cβ for the cloning of Vβ2 sequences to allow family desig-nation was: 5' ATGCTGCTGCTTCTGCTGCTTCTG 3'. Plasmid DNA extracted from white colonies was spotted into Hybond N$^+$ nylon membranes using a minifold slot blotter (model SRC 072; Schleicher & Schuell, Inc., Keene, NH) and hybridized with in-ternal Cα and Cβ ^{32}P-labeled oligonucleotides (probes A and B, Table 1). Sequence determination was performed on plasmid DNA from positive colonies. IPCR products were purified by phenol ex-traction, precipitated with ethanol, and digested with SalI and NotI (New England Biolabs Inc.). Fragments of expected sizes for the cDNAs (700 bp for β chain sequences) were separated by electropho-resis onto DEAE-cellulose membrane (NA-45 DEAE, Schleicher & Schuell, Inc.) through an agarose gel of the appropriate concen-tration. Recovered DNA fragments were ligated to phagescript vector arms (Stratagene, La Jolla, CA) obtained by SalI and NotI digestion. Phages were grown on *Epicurian coli* XL-1-Blue compe-tent cells (Stratagene). A total of 100,000–500,000 clones were ob-tained per microgram of RNA. Recombinant plaques were trans-ferred to nitrocellulose filters (Schleicher & Schuell, Inc.) as described (23). Membranes were hybridized with Cβ (probe B) or with oli-gonucleotides specific for either Vβ 2 (probe G) or for the specific β junctional region of Vβ2.1/Dβ1/Jβ1.1/Cβ1 TIL clones (probe D) as specified in Table 1. The frequency of Vβ2 gene usage was calculated from the ratio of the numbers of plaques positive with Vβ2 and Cβ oligonucleotides (G$^+$/B$^+$) and the frequency of TIL clone junctional region usage from the ratio of the numbers of plaques positive with the specific V/D/Jβ and Vβ2 probe (D$^+$/G$^+$). Single plaques were picked and grown up and recom-binant phage DNA was purified for DNA sequence determination.

Sequencing Reactions. Sequencing reactions were performed with Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH) by the dideoxy chain termination, procedure and N sequences were compared with gene data bank entries (GenBank, Los Alamos, NM; EMBL, Heidelberg, Germany) and to available published V (25–33) and J TCR gene segments (26–31, 34).

Oligonucleotides. All oligonucleotides used were prepared on an automated DNA synthesizer (model 380A; Applied Biosystems, Foster City, CA). Sequences of oligonucleotides used as hybridiza-tion probes, IPCR, and sequencing primers are shown in Table 1, whereas sequences of specific Vα1 → Vα22, Cα, Vβ1 → Vβ20, Cβ PCR primers are listed elsewhere (9, 21). For hybridization, oligonucleotides were endlabeled with [^{32}P]ATP (Amersham In-ternational) and T4-polynucleotide kinase (New England Biolabs Inc.). Hybridization and washing conditions in order to match their target sequence perfectly were chosen according to the length and GC content, as described by Sambrook et al. (23).

In particular, filters containing IPCR cDNA libraries were pre-hybridized for 5 h and then hybridized overnight at 42°C in a so-lution containing 3.2× SSC for probe D, 0.63× SSC for probe G, and 2.5× SSC for probe B. In addition, prehybridization and hybridization buffer contained 5× Denhardt's solution, 1% SDS, 50 mM Tris-HCl, pH 7.5, and 100 μg/ml salmon sperm DNA. ^{32}P-labeled oligonucleotide probes were added to the hybridization buffer. Filters were then washed twice at room temperature and twice at 42°C in a washing solution containing 0.1% SDS and the SSC concentration used for hybridization.

Results

TCR Vα and Vβ Usage by Fresh and Long-Term Cultured TIL and PBL from Patient 9742. MLTC lines TIL-1, -2, -3,

-4 and PBL-1, -2, -3, -4 were generated by culturing four independent aliquots of fresh TIL and PBL of patient 9742 with autologous melanoma cells (Me9742/2) and rIL-2. Each MLTC line was examined for TCR repertoire expression at day 28, day of cloning, and compared with uncultured TIL and PBL. This analysis was performed to see whether or not in vitro restimulation with autologous tumor is accompanied by oligoclonal expansion of T cells and to control for variability among multiple long-term cultures derived from the same pool of lymphocytes. An aliquot of either TIL or PBL, cultured for the same period of time in the presence of PHA, was included as control for polyclonal, Ag-independent stimulation. cDNAs were thus obtained from fresh TIL and PBL, from all MLTC and PHA lines, and were amplified by PCR for 30 cycles using Vα1 to Vα22 and Vβ1 to Vβ20 specific primers in combination with a Cα or a Cβ primer, respectively. Southern blots were hybridized with internal oligonucleotide probes for Cα (probe A, Table 1), Cβ (probe B, Table 1) in order to ascertain the specificity of amplification. The results obtained with fresh TIL (Fig. 1) indicated that their TCR Vα and Vβ repertoire was similar to that seen on fresh PBL (Fig. 2) and, as described in a preliminary report (35), not oligoclonal. Furthermore, no oligoclonality was found in either population after in vitro restimulation with autologous melanoma (Figs. 1 and 2) since most of Vα and Vβ families present in fresh lymphocytes were still expressed after 28 d of MLTC. However, differences in the intensity of the hybridization signals were consistently observed. Increase of the hybridization signal of Vα 5, 11, and 14, either absence or decrease in Vα 6, 9, 12, and 15 and Vβ4, 10, 11, 17, 18, and 19 could be detected in at least three out of four MLTC-TIL cultures (Fig. 1). Variations in MLTC-PBL cultures involved the increase of Vα14 and the decrease or absence of Vα15 and Vβ4, 10, and 17. A consistent increase of Vα2 was also observed in PBL-1, -3, and -4 (Fig. 2). The increase or decrease of the hybridization signals appeared to be associated with culture in the presence of autologous tumor since stimulation of TIL with PHA for the same period of time (28 d) failed to modify any of the above mentioned families and reflected the TCR Vα and Vβ usage of fresh TIL (Fig. 1). The MLTC lines from TIL and PBL, all expressing a predominant CD3$^+$, CD8$^+$, TCR-α/β$^+$ phenotype (data not shown), were also tested for cytotoxicity against two different autologous lesions (Me9742/2 and Me9742/1) and six allogeneic melanomas. All MLTC lines from TIL and at least two of the MLTC lines from PBL (PBL-1 and -2) displayed a preferential lysis of the two autologous metastatic cell lines and of the only HLA-A2–matched allogeneic melanoma (Me10538) (Table 2). TIL and PBL cultured with PHA and IL-2, but without autologous tumor cells, displayed a borderline cytotoxic activity on all targets.

Taken together, these data indicate that a preferential lysis of HLA-A2$^+$ melanomas can be the result of all MLTC from TIL and of at least two MLTC from PBL. This cytolytic specificity, however, was not associated with oligoclonality at the level of TCR. Therefore, we argued that only the analysis of CTL clones from the different MLTC could

Table 1. *Oligonucleotides Used in This Study*

Oligonucleotide	Sequence	Specificity	Purpose
A	5'-GGTACACGGCAGGGTCAGGGTTC	Cα	Hybridization probe/sequencing primer
B	5'-CTCGGGTGGGAACAC	Cβ	Hybridization probe/sequencing primer
C	5'-GTAGGTTAGTAGGCGCGC	V/J junctional region of Vα8.2/JαAP511/Cα TIL clones	Hybridization probe
D	5'-GAAAGCTTCACCGTCAAA	V/D/J junctional region of Vβ2.1/Dβ1/Jβ1.1/Cβ1 TIL clones	Hybridization probe
E	5'-GGGTCGACCTGTGCACCTCCTTCCCATT	Cβ	IPCR forward primer
F	5'-GCATGCGGCCGCATGGCCATGGTCAAGAGA	Cβ	IPCR inverse primer
G	5'-AGGTCAGGCTTGCATGGTTGATGA	Vβ2	Hybridization probe

Vα

Vβ

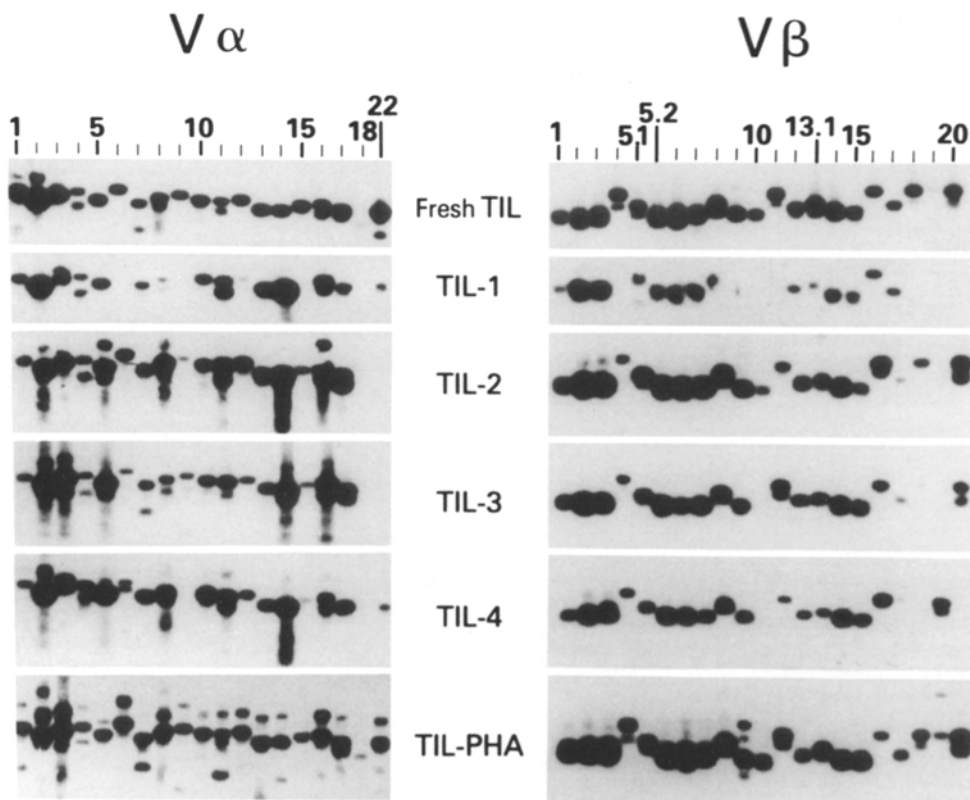**Figure 1.** TCR Vα and Vβ repertoire of fresh TIL, of day 28 MLTC from TIL (TIL-1, -2, -3, -4) and of day 28 PHA-activated TIL, as detected by Southern blot analysis of PCR-amplified cDNA with TCR Vα (1–22) -Cα and Vβ (1–20) -Cβ primers. Hybridization was performed with Cα (probe A, Table 1) or Cβ (probe B, Table 1) internal oligonucleotides.

establish the correlation between functional specificity of the effectors and the structure of the corresponding TCR.

TCR Vα and Vβ Usage by Antimelanoma T Cell Clones. From all TIL and PBL lines previously analyzed, a total of 112 clones with a *p* value for clonality >0.95 were derived. Of these clones, 38 were selected for TCR Vα/Vβ analysis (24 clones from TIL and 14 from PBL) on the basis of (*a*) cytotoxic activity on the autologous tumor, and (*b*) inhibi-

tion of lysis by anti-CD3 and anti-HLA class I mAb. A T cell clone (clone 119), obtained by direct cloning of fresh PBL with PHA, was also included in the analysis. All 39 CTL clones expressed a CD3$^+$, CD4$^-$, CD8$^+$, TCR-α/β$^+$ phenotype (data not shown) and, as shown in Table 3, displayed cytotoxic activity on the autologous melanoma with no or borderline activity on K562. Lysis of autologous melanoma by all these clones was specifically blocked by anti-CD3
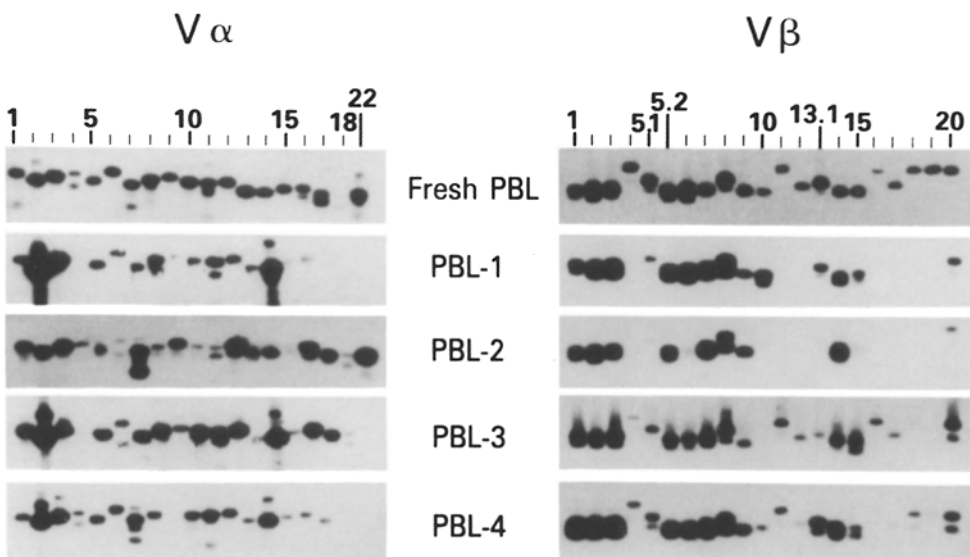
Vα

Vβ

Figure 2. TCR Vα and Vβ repertoire of fresh PBL and of day 28 MLTC from PBL (PBL-1, -2, -3, -4) as detected by Southern blot analysis of PCR-amplified cDNA with TCR Vα (1–22) -Cα and Vβ (1–20) -Cβ primers. Hybridization was performed with Cα (probe A, Table 1) or Cβ (probe B, Table 1) internal oligonucleotides.

Table 2. *Lysis of Autologous (9742/2, 9742/1) and Allogeneic Melanomas by TIL and PBL Cultures at day 28 of MLTC*

Effectors	9742/2*	9742/1*	665/1	1402	10538*	4405	15392	1007
TIL-1	1249‡	812	98	291	2375	84	11	<1
TIL-2	861	459	123	26	491	112	8	<1
TIL-3	1846	1252	89	24	1569	36	19	<1
TIL-4	744	388	<1	<1	324	<1	<1	<1
TIL-PHA	2	3	124	24	<1	53	3	<1
PBL-1	1077	1053	613	569	1069	447	245	21
PBL-2	2051	1691	556	652	2211	553	158	6
PBL-3	3793	2065	2462	3359	1997	1926	1357	367
PBL-4	1974	2104	970	1267	3198	1065	619	239
PBL-PHA	<1	<1	19	8	12	14	1	<1

Lysis of autologous metastatic lesions (9742/2 and 9742/1) and of allogeneic melanomas (665/1, 1402, 10538, 4405, 15392, and 1007) by MLTC from TIL (−1, −2, −3, −4) and from PBL (−1, −2, −3, −4) and by PHA-activated TIL and PBL was tested in a 4-h ^{51}Cr-release assay at four different E/T ratios ranging from 50:1 to 0.4:1.
* HLA-A2$^+$ melanoma cell lines.
‡ Results of cytotoxic assays are expressed as LU/10^7 cells.

Table 3. *Lytic Activity and TCR V Gene Usage of 24 TIL Clones and of 15 PBL Clones*

MLTC	CTL Clone	9742/2	9742/2 + anti-CD3*	9742/2 + anti-HLA*	K562	TCR‡ Vα, Vβ
TIL-1	A21	43§	12	13	12	8, 2
	A26	41	13	4	12	8, 2
	A16	35	12	15	14	8, 2
	A27	34	10	15	8	8, 2
	A14	34	5	9	10	8, 2
	A24	33	8	16	10	8, 2
	A2	32	9	20	16	8, 2
	A18	29	11	18	17	8, 2
	A23	27	4	14	8	8, 2
	A8	27	7	8	10	8, 2
	A3	27	9	12	15	8, 2
	A37	26	7	8	11	8, 2
	A36	24	3	2	5	8, −
	A39	23	8	8	9	8, 2
	A5	22	4	6	10	8, 2
	A25	43	11	11	11	14, 2
TIL-2	A48	25	1	2	6	8, 2
	A50	24	0	8	16	8, 2

Table 3. *(continued)*

MLTC	CTL Clone	Targets				TCR[‡] Vα, Vβ
		9742/2	9742/2 + anti-CD3*	9742/2 + anti-HLA*	K562	
	A58	24	<u>1</u>	<u>8</u>	6	8, 2
	A44	19	<u>2</u>	<u>5</u>	10	8, 2
	A54	15	<u>0</u>	<u>4</u>	13	8, 2
TIL-3	A62	16	<u>1</u>	<u>5</u>	7	8, 2
TIL-4	A72	32	<u>10</u>	<u>15</u>	11	8, 2
	A70	31	<u>4</u>	<u>12</u>	6	8, 2
PBL-1	A96	54	<u>35</u>	<u>28</u>	4	2, 13.2[¶]
	A100	31	7	20	8	2, 13.2[¶]
	A89	23	<u>2</u>	<u>1</u>	2	2, 13.2[¶]
	A81	40	<u>8</u>	<u>22</u>	4	2, 14
	A103	49	<u>21</u>	<u>25</u>	7	2, 14
	A94	47	<u>18</u>	<u>27</u>	7	2, 14
	A83	43	<u>17</u>	<u>22</u>	12	2, 14
	A91	43	<u>7</u>	<u>19</u>	6	2, 14
	A109	40	<u>6</u>	<u>9</u>	5	2, 14
	A80	30	<u>3</u>	<u>4</u>	6	2, 14
	A75	33	<u>4</u>	<u>8</u>	2	2, 14
	A78	33	<u>3</u>	<u>5</u>	4	2 6, 3 14
	A93	25	<u>3</u>	<u>3</u>	2	8, 2
PBL-4	A147	21	<u>5</u>	<u>11</u>	17	8, 2
PBL-PHA		119	35	<u>5</u>	<u>8</u>	5	2, w22[¶]

CTL clones isolated by cloning lymphocytes from MLTC TIL-1, -2, -3, and -4, PBL-1 and -4, and PHA-activated PBL were tested for lysis of autologous tumor (9742/2) and of K562 cells at the E/T ratio of 10:1 in a 4-h ^{51}Cr-release assay.

* Inhibition of lysis of autologous tumor was tested after preincubation of CTL clones with anti-CD3 mAb (OKT3) or of the tumor target with anti-HLA class I mAb (w6/32).

‡ TCR Vα and Vβ expression of each clone was determined by PCR as described in Material and Methods.

§ Results expressed as percent lysis. Underlined values represent significant inhibition of lysis in comparison to lysis of 9742/2 in the absence of Abs (SNK test, $p = 0.01$).

¶ TCR Vβ expression of each clone was determined by IPCR as described in Material and Methods.

(OKT3) and by anti-MHC class I (W6/32) mAbs (Table 3). The Vα and Vβ gene segments utilized in TIL and PBL clones were examined by PCR, and the results are also shown in Table 3. In agreement with the statistical prediction of clonality, only one α and β rearranged transcript could be detected in all but one (PBL-1 clone A78) of the clones studied. The presence of two α and β chain transcripts in clone A78 is consistent with the hypothesis that it may not represent a true clone and, for this reason, it has not been further investigated. The variable β chain expressed by PBL-1 clones A96, A100, and A89 and by the PBL-PHA clone 119 was

identified by IPCR since no amplification could be detected with the Vβ primers used.

All T cell clones derived from TIL-1, -2, -3, and -4 expressed a TCR composed by Vα8 and Vβ2 V region genes (Table 3), despite the fact that TIL clones were selected from four independent MLTC cultures, except for TIL-1 clones A25 (Vα14/Vβ2) and A36, for which only the α chain (Vα8/non Vβ2) could be fully investigated because of the low amount of starting material. Moreover, PBL clones A93 and A147 derived from two independent MLTC cultures (PBL-1 and -4) expressed the same Vα8 and Vβ2 transcripts

A

Clone	MLTC	Vα	Vα	CDR3	Jα	Cα
A21	TIL-1	Vα8.2/JαAP511	TGTGCAGCG / C A A	CGCCTACTAACTACAAATAC / R L L T Y K Y	ATCTTTGGAACAGGCACCAGGCTGAAGGTTTTAGCA / I F G T G T R L K V L A	AATATCCAG / N I Q
A50	TIL-2	Vα8.2/JαAP511	TGTGCAGCG / C A A	CGCCTACTAACTACAAATAC / R L L T Y K Y	ATCTTTGGAACAGGCACCAGGCTGAAGGTTTTAGCA / I F G T G T R L K V L A	AATATCCAG / N I Q
A62	TIL-3	Vα8.2/JαAP511	TGTGCAGCG / C A A	CGCCTACTAACTACAAATAC / R L L T Y K Y	ATCTTTGGAACAGGCACCAGGCTGAAGGTTTTAGCA / I F G T G T R L K V L A	AATATCCAG / N I Q
A70	TIL-4	Vα8.2/JαAP511	TGTGCAGCG / C A A	CGCCTACTAACTACAAATAC / R L L T Y K Y	ATCTTTGGAACAGGCACCAGGCTGAAGGTTTTAGCA / I F G T G T R L K V L A	AATATCCAG / N I Q
A93	PBL-1	Vα8.2/JαAP511	TGTGCAGCG / C A A	CGCCTACTAACTACAAATAC / R L L T Y K Y	ATCTTTGGAACAGGCACCAGGCTGAAGGTTTTAGCA / I F G T G T R L K V L A	AATATCCAG / N I Q
A147	PBL-4	Vα8.2/JαAP511	TGTGCAGCG / C A A	CGCCTACTAACTACAAATAC / R L L T Y K Y	ATCTTTGGAACAGGCACCAGGCTGAAGGTTTTAGCA / I F G T G T R L K V L A	AATATCCAG / N I Q
A36	TIL-1	Vα8.1/JαV	TGTGCAGCT / C A A	CATTCCTCTGGGGCTGGGAGTTACCAACTC / H S S G A G S Y Q L	ACTTTCGGGAAGGGGACCAAACTCTCGGTCATACCA / T F G K G T K L S V I P	
A25	TIL-1	Vα14	TGTGCCGTG / C A V			
A96	PBL-1	Vα2.1/JαIGRJα10	TGTGCCGTG / C A V	AACAAAGGCAATGACATG / N K G N D M	CGCTTTGGAGCAGGGACCAGACTGACAGTAAAACCA / R F G A G T R L T V K P	AATATCCAG / N I Q
A81	PBL-1	Vα2.1/JαIGRJα04	TGTGCCGTG / C A V	AATACTGGAGGCTTCAAACT / N T G G F K T	ATCTTTGGAGCAGGAACAAGACTATTGTTAAAGCA / I F G A G T R L L F V K A	AATATCCAG / N I Q
119	PBL-PHA	Vα2.1/JαT	TGTGCCGTG / C A V	AAGGGGATATCAGGAGGAAGCTACATAACCT / K G I S G G S Y I P	ACATTCGAAGAGAACCAGCCTTATTGTTCATCCG / T F G R G T S L I V H P	TATATCCAG / Y I Q

Figure 3. TCR α (A) and β (B) cDNA junctional nucleotide sequences of independent melanoma-specific TIL and PBL clones. A translated amino acid sequence (in the single letter code) is shown under each corresponding nucleotide sequence. For clones derived from the same MLTC only different TCR sequences are reported. PBL-1 clones A103, A94, A83, A91, A109, A80, A75 and PBL-1 clones A100 and A89, all share an identical nucleotide sequence as, respectively, clone A81 and A96. Sharing of the same TCR by the remaining Vα8/Vβ2 TIL clones has been assessed by hybridization with oligonucleotides specific for the V/J α and V/D/J β junctional region as specified in the text. V gene segments were classified according to family designations outlined by Wilson et al. (25). Vα chain sequences Vα8.2 (HAP50), Vα8.1 (HAP41) and Vα2.1 (HAP26) are originally reported by Yoshikai et al. (27). Jα sequences were assigned to previously described Jα gene segments according to the following authors: Klein et al. (26), JαT; Yoshikai et al. (27), JαV; and Roman-Roman et al. (29), IGRJα10, IGRJα04. JαAP511 differ from the original report (26) by a point mutation that

B

Clone	MLTC	Vβ	CDR3	Jβ	Cβ
A21	TIL-1	Vβ2.1/Dβ1/Jβ1.1/Cβ1	TGCAGTGCT (C S A)	TTTGACGGTGAAGCT (F D G E A)	TTCTTTGGACAAGGCACCAGACTCACAGTTGTA (F F G Q G T R L T V V)	GAGGACCTG (E D L)
A50	TIL-2	Vβ2.1/Dβ1/Jβ1.1/Cβ1	TGCAGTGCT (C S A)	TTTGACGGTGAAGCT (F D G E A)	TTCTTTGGACAAGGCACCAGACTCACAGTTGTA (F F G Q G T R L T V V)	GAGGACCTG (E D L)
A62	TIL-3	Vβ2.1/Dβ1/Jβ1.1/Cβ1	TGCAGTGCT (C S A)	TTTGACGGTGAAGCT (F D G E A)	TTCTTTGGACAAGGCACCAGACTCACAGTTGTA (F F G Q G T R L T V V)	GAGGACCTG (E D L)
A70	TIL-4	Vβ2.1/Dβ1/Jβ1.1/Cβ1	TGCAGTGCT (C S A)	TTTGACGGTGAAGCT (F D G E A)	TTCTTTGGACAAGGCACCAGACTCACAGTTGTA (F F G Q G T R L T V V)	GAGGACCTG (E D L)
A93	PBL-1	Vβ2.1/Dβ1/Jβ1.1/Cβ1	TGCAGTGCT (C S A)	TTTGACGGTGAAGCT (F D G E A)	TTCTTTGGACAAGGCACCAGACTCACAGTTGTA (F F G Q G T R L T V V)	GAGGACCTG (E D L)
A147	PBL-4	Vβ2.1/Dβ1/Jβ1.1/Cβ1	TGCAGTGCT (C S A)	TTTGACGGTGAAGCT (F D G E A)	TTCTTTGGACAAGGCACCAGACTCACAGTTGTA (F F G Q G T R L T V V)	GAGGACCTG (E D L)
A36	TIL-1	N.D.				
A25	TIL-1	Vβ2.1/Dβ1/Jβ1.2/Cβ1	TGCAGTGCT (C S A)	AAGTCCAGGGTTTATGGCTAC (K S R V Y G Y)	ACCTTCGGTTCGGGGACCAGGTTAACCGTTGTA (T F G S G T R L T V V)	GAGGACCTG (E D L)
A96	PBL-1	Vβ13.2/Dβ1/Jβ1.2/Cβ1	TGTGCCAGC (C A S)	AGTTACTCTGCGGCGTCGGGTCATGCTAC (S Y S A A S G H G Y)	ACCTTCGGTTCGGGGACCAGGTTAACCGTTGTA (T F G S G T R L T V V)	GAGGACCTG (E D L)
A81	PBL-1	Vβ14/Dβ1/Jβ1.2/Cβ1	TGTGCCAGC (C A S)	AGCACGGGACAGGGGGGTGGGCCTCC (S T G Q G W G S)	TTCGGTTCGGGGGACCAGGTTAACCGTTGTA (F G S G T R L T V V)	GAGGACCTG (E D L)
119	PBL-PHA	Vβw22/Dβ2/Jβ2.1/Cβ2	TGTGCCAGC (C A S)	AGGTCTAGCAGGACGAGGAGCAG (R S S R E E Q)	TTCTTCGGGCCAGGGACACGGCTCACCGTGCTA (F F G P G T R L T V L)	GAGGACCTG (E D L)

results in the amino acid Y (*double underline*). This JαAP511 variant has been recently reported by others (24). Vβ chain sequences Vβ2.1 (PL2.13) and Vβ14 (PL8.1) were originally reported by Concannon et al. (30), Vβ13.2 (HBP34) by Kimura et al. (31), and Vβw22 (IGRβ03) by Ferradini et al. (33). Dβ, Jβ, and Cβ elements are assigned according to Toyonaga et al. (34). For each clone, only the last 3 V gene residues are shown, followed by the presumed Ig-like loops (CDR3) defined according to Chothia et al. (36) and by the first three residues of the C region. Cβ usage was established from the partial Cβ sequences obtained. For clones A36 and A25, only α or β chain, respectively, could be sequenced because of the low amount of cDNA available. The EMBL accession numbers for TCR α and β chains of clones are: X74392, X74393 (A21, A50, A62, A70, A93, A147); X74396, X74397 (A96); X74394, X74395 (A81); X74390, X74391 (119); X74398 (TCR α chain of A36); X74399 (TCR β chain of A25).

(Table 3). All other T cell clones, derived from PBL, preferentially used Vα2 joined to either Vβ13.2, Vβ14, or Vβw22 (Table 3). No clones expressing Vα8 and only two clones expressing Vβ2 were found in 15 TIL clones derived from the same patient and lacking specificity for the autologous melanoma (data not shown).

Sequencing of TCR α and β cDNAs of TIL and PBL Clones. Nucleotide sequence of TCR α and β cDNAs indicated that Vβ8/Vβ2 clones A21, A50, A62, A70, A93, and A147 derived from the four independent in vitro MLTC cultures of TILs and from two independent cultures of PBLs, share exactly the same TCR. In fact, as shown in Fig. 3, not only did they use the same Vα and Vβ family members, namely Vα8.2 (27) and a Vβ2.1 (30) recently designed as Vβ2.1a by Plaza et al. (32), but they had identical α and β V/(D)/J junctional sequences. Vα8.2 was rearranged to a Jα segment identical to the published JαAP511 segment (26) except for a single base substitution, whereas Vβ2.1 was rearranged to Dβ1, Jβ1.1, and Cβ1 (34). Dβ1 segment was assigned on the basis of requirements for gene rearrangement since the exact identification of this region was impossible, due both to junctional and N region diversification and to the short length of the germline segment (25). All remaining Vα8/Vβ2 TIL clones did share the same junctional region as assessed by hybridization of Southern blots containing Vα8-Cα or Vβ2-Cβ rearranged bands with oligonucleotides C and D (Table 1), specific for the junctional region of Vα8.2/JαAP511/Cα or Vβ2/Dβ1/Jβ1.1/Cβ1 chains, respectively (data not shown). Two other TCR configurations were identified by sequence analysis of PBL-1 clones, in addition to that composed by Vα8/Vβ2. In their α chain, usage of

the same V family member, Vα2.1 (27) and presence of N region nucleotides at the V/J junction that create codons for a shared amino acid could be observed, whereas the β chain included Vβ13.2 (31) and Vβ14 (30) that belong to a subgroup of structurally related Vβ families (36) and the same Jβ1.2 gene segments. Apart from these similarities, both α and β chain junctional regions are different in sequence and length. PBL-PHA clone 119 shared with PBL-1 clones the usage of Vα2.1 but employed the recently described Vβ w22 region (33) and was highly diverse in junctional sequences.

No predominant structural features were present when sequence of dominant TIL clones was compared with that of other TIL or PBL clones. As summarized in Table 4, only six different functional TCR are found among melanoma-specific and HLA class I–restricted T cell clones derived from TIL or PBL of a melanoma patient.

The Vα8.2/Vβ2.1⁺ and Vα2.1/Vβ14⁺ Clones from TIL and PBL Are HLA-A2 Restricted and Recognize Distinct Melanocyte-Lineage–Specific Ags. PBL clones A75, A81, A83, A94, and A103 (sharing a Vα2.1/IGRJα04/Cα - Vβ14/Dβ1/Jβ1.2/Cβ1 TCR, Fig. 3) and TIL clones A37 and A54 (sharing a Vα8.2/JαAP511/Cα - Vβ2.1/Dβ1/Jβ1.1/Cβ1 TCR (Fig. 3) have been recently shown to be HLA-A2 restricted and to recognize Ags expressed on autologous melanoma cells and on HLA-A2–matched normal melanocytes (15). Moreover, specificity analysis of PBL clone A81 revealed that the antigen recognized was present on the majority of HLA-A2 allogeneic melanomas (15). We thus investigated whether TIL clone A21, representative of the dominant TCR found in TIL (i.e., Vα8.2/JαAP511/Cα - Vβ2.1/Dβ1/Jβ1.1/Cβ1), expressed a similar or different pattern of lytic

Table 4. *Summary of TCR α and β Chain Composition in Melanoma-specific CTL Clones Isolated from MLTC of TIL and PBL and from PHA-activated PBL*

CTL clones isolated from	TCR composition*					
	Vα	Jα	Vβ	Jβ	Cβ	Frequency[†]
TIL-1	8.2	AP511	2.1	1.1	1	14/16
TIL-2	8.2	AP511	2.1	1.1	1	5/5
TIL-3	8.2	AP511	2.1	1.1	1	1/1
TIL-4	8.2	AP511	2.1	1.1	1	2/2
PBL-1	8.2	AP511	2.1	1.1	1	1/12
PBL-4	8.2	AP511	2.1	1.1	1	1/1
TIL-1	8.1	JαV				1/16
TIL-1	14		2.1	1.2	1	1/16
PBL-1	2.1	IGRJα10	13.2	1.2	1	3/12
PBL-1	2.1	IGRJα04	14	1.2	1	8/12
PBL-PHA	2.1	JαT	w22	2.1	2	1/1

* Nomenclature and references for the Vβ, Jβ, Vα, and Jα gene segments are in the legend to Fig. 3.
† No. of clones expressing the given TCR composition/No. of clones examined from each MLTC or from PHA-activated lymphocytes.

activity in comparison with the Vα2.1/IGRJα04/Cα - Vβ14/Dβ1/Jβ1.2/Cβ1 PBL clone A81. As shown in Table 5, cytolysis of the autologous melanoma by clones A21 and A81, but not by LAK cells (data not shown), was blocked by mAb CR.11.351, indicating that MHC molecule HLA-A2 is involved in the recognition of melanoma Ag(s) by T cell clones bearing these types of TCR. Moreover, in agreement with recognition of Ags expressed in vivo, both clones lysed the fresh tumor. However, whereas clone A81 recognized all allogeneic HLA-A2+ melanomas, the TIL clone A21 lysed significantly only four out of seven HLA-A2+ melanomas. No lysis of HLA-A2− melanomas was observed by either clone. Furthermore, confirming the previous results obtained with T cell clones A75, A83, and A94 (15), CTL A81 and A21 lysed the two HLA-A2+ normal melanocytes (FM741 and FM727) but not two HLA-A2− melanocytes (FM713 and FM216/a). In addition, neither clone lysed any normal or neoplastic cells of nonmelanocyte lineage, including the autologous lymphoblastoid cell line from patient 9742, in a panel of 17 targets, even though 11 targets were HLA-A2+ (Table 5). Only a borderline reactivity was seen by clone A21 on one HLA-A2+ fibroblast line (F1338/1). Taken together, these data suggest that TIL and PBL clones, expressing two different TCR, define distinct antigenic determinants preferentially expressed in cells of the melanocyte lineage. Both Ags are recognized in association with the HLA-A2 restriction element but are distinguishable on the basis of their broad or more restricted distribution on allogeneic HLA-A2+ melanomas.

Concerning the other clones bearing different TCR, PBL clone 119 was not HLA-A2 restricted and was shown to recognize an antigenic determinant in association with a different, yet unidentified HLA class I allele (15). TIL clones A36 and A25 and PBL clones A96, A100, and A89 could not be tested because of the insufficient amount of cells obtained to perform the specificity assay.

The V/D/J Sequence Associated with the Dominant HLA-A2–restricted TIL Clone Is Expressed More in the Tumor Sample and Fresh TIL than in PBL. The identification from distinct TIL cultures of the same dominant clone characterized by a Vα8.2/JαAP511/Cα–Vβ2.1/Dβ1/Jβ1.1/Cβ1 TCR suggests, but does not prove, that a selective in vivo expansion/accumulation of this T cell occurred before the in vitro activation of the lymphocytes. To address this point, we screened cDNA libraries, constructed by IPCR using RNA extracted directly from the Me9742/2 surgical specimen, from freshly isolated TIL and from PBL, for β chain expression with specific oligonucleotide probes. Positive plaques for each oligonucleotide were then counted. Hybridization with an upstream Cβ probe (probe B, Table 1) showed that >80% of the clones contained TCR-β sequences. As shown in Table 6, the Vβ2 family sequences, as detected by hybridization with a Vβ2-specific oligonucleotide (probe G, Table 1), represented 10.2 and 11.7% of the total β chains in tumor samples and in TIL, respectively, vs. 6.5% in PBL. These results are in a similar range of frequency detected by two-color immunofluorescence followed by FACS® analysis using a com-

mercially available anti-Vβ2 mAb (mAb TCR Vβ2 No. 1149; Immunotech, Luminy, France) in association with a PE-conjugated anti-CD3 mAb (LEU-4PE No. 7347; Becton Dickinson & Co.). This analysis indicated that 9% of CD3+ cells in PBL and 9.5% of CD3+ cells in TIL were positive for Vβ2 expression (data not shown). However, the β chain sequence corresponding to the junctional region of the dominant T cell clone was strikingly overrepresented in the tumor infiltrate. In fact, 19 and 18.4% of all Vβ2+ sequences in the surgical specimen and in the uncultured purified TIL extracted from the same sample were positive with oligonucleotide D. By contrast, no hybridizing clones were detected among 520 Vβ2+ cDNA clones screened from the PBL sample. This indicates that the frequency of this specific sequence in the PBL had to be much lower than in the tumor sample and at least lower than 0.19% (1/520 = 0.00192) of all Vβ2+ sequences expressed in the PBL. In addition, all cDNA clones positive for oligonucleotide D were comprised in the Vβ2+ β chains and sequence analysis of five random oligonucleotide D+ cDNA clones confirmed presence of the expected sequence (data not shown). To find out whether or not the Vβ2+ sequences that did not hybridize with oligonucleotide D were oligoclonal, the primary structure of 10 randomly selected Vβ2+ cDNA clones derived from TIL was determined. All TCR Vβ2 chains sequenced were in frame and showed no evidence of clonality since none of the 10 sequences examined was repeated (data not shown).

These results indicate that the sequence corresponding to the specific TCR β chain expressed by an in vitro dominant TIL clone is preferentially expressed at the site of tumor growth in comparison to peripheral blood. This finding is consistent with the hypothesis that this T cell clone may have been subjected, in vivo, to a selective expansion/accumulation driven by an HLA-A2–associated melanocyte-lineage–specific Ag.

Discussion

The present study was aimed at the molecular characterization of TCR expressed by fresh and cultured TIL and PBL as well as by CTL clones isolated from MLTC of TIL and PBL of an HLA-A2+ melanoma patient in relation to their fine specificity and in vivo relevance. The analysis of TCR repertoire performed on fresh TIL indicated that the degree of diversity of this population of lymphocytes was similar to that found in circulating PBL. Furthermore, the same analysis performed on independent MLTC revealed that after 28 d of culture, both TIL and PBL were still as heterogeneous as uncultured cells in their TCR repertoire. This was in contrast with the emergence of a preferential lytic activity against autologous or allogeneic HLA-A2+ melanomas observed not only in all the TIL cultures, but also in at least two MLTC from PBL. These findings suggested that it was not possible to link any of the different TCR expressed in these cultures with the preferential lytic activity. The panel of clones derived from MLTC of TIL and PBL provided the opportunity to correlate TCR structure with antigenic specificity. Despite the large number of TCR Vα and Vβ families expressed

Table 5. *Specificity of PBL Clone A81 (Vα2.1/Vβ14 TCR) and TIL Clone A21 (Vα8.2/Vβ2.1 TCR)*

Target	Histology	HLA-A2 expression*	Effectors A81	Effectors A21
Me9742/2	Metastatic melanoma	96	33‡	20
Me9742/2	Metastatic melanoma	96	6§	0§
Fresh Me9742/2	Metastatic melanoma	96	20	13
Me13294	Metastatic melanoma	95	18	3
Me18732	Metastatic melanoma	95	27	5
Me8959	Metastatic melanoma	95	24	0
Me16938	Metastatic melanoma	93	65	39
Me3046/2	Metastatic melanoma	92	50	26
Me14932	Metastatic melanoma	94	42	9
Me10538	Primary melanoma	96	11	23
Me1402/R	Primary melanoma	0	0	0
Me13443	Metastatic melanoma	0	0	0
Me4024	Metastatic melanoma	0	0	3
Me665/1	Metastatic melanoma	0	3	6
Me1340	Metastatic melanoma	0	0	0
Me1811	Metastatic melanoma	4	0	2
FM216/a	Normal melanocyte	0	1	0
FM713	Normal melanocyte	0	0	2
FM741	Normal melanocyte	98	27	8
FM727	Normal melanocyte	96	40	20
Reca458	Renal carcinoma	82	1	6
RecaSor	Renal carcinoma	93	0	2
RecaMar	Renal carcinoma	17	0	5
HT29	Colon carcinoma	0	2	1
A431	Epidermoid carcinoma	0	1	5
SKBR3	Breast carcinoma	13	0	7
CALU3	Lung carcinoma	91	4	0
Ovca432	Ovarian carcinoma	2	0	0
Skov3	Ovarian carcinoma	91	0	1
Igrov1	Ovarian carcinoma	0	0	6
N592	Small cell lung carcinoma	10	2	3
H446	Small cell lung carcinoma	0	0	2
Re458	Normal kidney cells	83	0	6
F1338/1	Fibroblasts	86	1	10
LCL9742	EBV-transformed B cells	95	2	0
LCL10538	EBV-transformed B cells	85	0	0
LCL1811	EBV-transformed B cells	2	2	0

Specificity of CTL clones A81 and A21 from patient 9742 was tested in a 4-h ^{51}Cr-release assay at the E/T ratio of 10:1.

* Reactivity of different cells with the mAb CR11.351 (anti-HLA-A2) is expressed as percent positive cells by cytofluorimetric analysis. All targets in the panel were positive for HLA-class I antigens as detected by the mAb w6/32 (data not shown).

‡ Results expressed as percent lysis. Underlined values are significantly different (SNK test, $p = 0.01$) from spontaneous release of each target. Results of the lysis of autologous melanoma and on melanocytes by A81 were already published (15, Table 2) but are listed again for a clearer comparison with the lytic activity of clone A21.

§ Lysis in the presence of 1 μg/ml of anti-HLA-A2 mAb CR11.351 was significantly different from lysis of the same target in the absence of Ab. LCL9742, LCL10538, and LCL1811 are lymphoblastoid cell lines autologous, respectively, to the tumors Me9742, Me10538, and Me1811. Re458 kidney cells are autologous to the tumor Reca458. FM216/a, FM713, FM741, and FM727 are normal melanocytes isolated from human neonatal foreskin. The biological characteristics and phenotype of melanocytes were described (15).

Table 6. *Frequency of V$\beta2^+$ and V$\beta2.1$/D$\beta1$/J$\beta1.1$/C$\beta1$ Junctional Region-positive Sequences in the Surgical Specimen, in Fresh Uncultured TIL from the Same Specimen, and in PBL of Melanoma Patient 9742*

Source of RNA	No. of V$\beta2^+$ plaques Total No. of Cβ^+ plaques	No. of V$\beta2.1$/D$\beta1$/J$\beta1.1$/C$\beta1$ junctional region-positive plaques Total No. of V$\beta2^+$ plaques
Surgical specimen	300/2950 (10.2%)	57/300 (19%)
Fresh TIL	245/2096 (11.7%)	45/245 (18.4%)
Fresh PBL	520/8000 (6.5%)	0/520

cDNA libraries generated by IPCR with the total RNA extracted from the surgical specimen, from uncultured TIL and from PBL were plated and hybridized with oligonucleotides B (Cβ), G (V$\beta2$), and D (V/D/J junctional region of V$\beta2.1$/D$\beta1$/J$\beta1.1$/C$\beta1^+$ TIL clones). No. of plaques positive for each oligonucleotide probe are shown and the percent frequency of the corresponding gene segment is expressed as in parentheses.

in independent MLTC, the majority of TIL clones as well as two independently derived PBL clones, were clonotypic, sharing not only identical V$\alpha8.2$ and V$\beta2.1$ chains but also identical J and/or D regions. Studies performed with cloned T cells (37) as well as by limiting dilution analysis (38) have demonstrated that specific antitumor CTL precursors are present in PBL and in TIL of cancer patients. However, when the antigenic specificity is similar, the knowledge of the molecular composition of the TCR is mandatory to assess whether or not specific CTL clones found in PBL or in TIL represent copies of the same T cell. The evidence described in this study, namely that the same T cell clone may be found in the periphery and at the tumor site but with different frequency, is consistent with the possibility that tumor-associated Ags can induce a bias in the tissue distribution of T cells recognizing them.

The analysis of the TCR structure of different T cell clones isolated from the TIL or PBL of patient 9742 indicated that the HLA-A2–restricted cytolytic response to the tumor is mediated by at least two different TCR. The comparison of antigenic specificity of HLA-A2–restricted clones expressing these different TCR (PBL clone A81, bearing a V$\alpha2.1$/V$\beta14$ TCR and TIL clone A21, bearing a V$\alpha8.2$/V$\beta2.1$ TCR) confirms the results recently obtained with other clones from the same patient (15) and indicates that these effectors can recognize Ags expressed not only on autologous or allogeneic HLA-A2$^+$ melanomas, but also on normal HLA-A2$^+$ melanocytes. Furthermore, the antigenic determinants recognized by the two different CTL clones were expressed with different frequency among HLA-A2$^+$ melanomas. These findings, therefore, are consistent with the hypothesis that the HLA-A2 molecule may act as a restricting element for more than one melanocyte-lineage–associated Ag and that distinct HLA-A2–peptide complexes are recognized by different TCR. Further evidence supporting the hypothesis that the antigenicity of a human melanoma is based on a number of antigenic determinants recognized by distinct TCR, was provided by the analysis of the TCR expressed by the non-HLA-A2–restricted CTL clone 119 (TCR V$\alpha2.1$/Vβw22), whose specificity has been recently described (15). This result rules out any corre-

lation between TCR V$\alpha2.1$ usage and the restricting HLA-A2 molecule. Although six different TCR are expressed by the specific CTL clones characterized in this study, because of an insufficient number of cells, no information is available on the specificity of TIL clones A25 and A36 and of PBL-1 clones characterized by V$\alpha2.1$/V$\beta13.2$ TCR that share some structural features with V$\alpha2.1$/V$\beta14$ TCR.

The information on the TCR structure of the CTL clones allowed us to correlate the antigenic specificity (namely HLA-A2–restricted recognition of a melanocyte-lineage–associated Ag) of the dominant category of TIL effectors with the relative frequency, in vivo, of the specific TCR β chain sequence expressed by the same CTL clones. To analyze whether antitumor T cells expressing TCR gene segments that characterize dominant TIL clones were enriched in lymphocytes obtained directly from the tumor infiltrate, as compared with PBL, we hybridized cDNA libraries generated by IPCR with specific oligonucleotides for either TCR V$\beta2$ or the junctional β region of the dominant TIL clone. By this technique we could show that the specific junctional V$\beta2.1$/D$\beta1$/J$\beta1.1$ sequence represented 19 and 18.4% of all V$\beta2^+$ cDNA clones in fresh TIL or in the surgical tumor specimen, respectively. By screening 520 V$\beta2^+$ cDNA plaques derived from PBL libraries similarly constructed, no positive plaques could be detected, indicating that the frequency of this sequence had to be much lower in PBL than in TIL and at least lower than 0.19% (1/520) of the V$\beta2^+$ sequences found in PBL. Interestingly, we found evidence that clones expressing this specific Vβ sequence were not absent from the peripheral circulation, as shown by the observation that two replicates of an effector bearing the specific V$\beta2.1$/D$\beta1$/J$\beta1.1$/Cβ TCR β chain were present among PBL clones isolated from the MLTC PBL-1 and PBL-4.

Taken together, the results obtained by the IPCR technique are consistent with the hypothesis that the expression on melanoma of a melanocyte-related antigen can alter the tissue distribution of a T cell clone recognizing that Ag. This process may be due to a phenomenon of progressive and selective accumulation of cells from the same circulating T cell clone at the site of tumor growth. This may have led to a

progressive reduction in the relative abundance of the specific $V\beta2.1/D\beta1/J\beta1.1^+$ TCR β sequences in the population of mRNA extracted from the PBL or, in other words, to a reduction in the frequency of the corresponding clone in the circulating pool. Alternatively, our results may also be explained assuming that one or more replicates of the TCR $V\beta2.1/D\beta1/J\beta1.1^+$ CTL clone underwent a phase of clonal expansion at the site of tumor growth, possibly as the result of Ag recognition. Both these processes can also explain the observation that the same CTL clone was found as independent replicates in distinct MLTC of TIL, a finding suggesting that the same T lymphocyte was present in multiple copies in the freshly purified TIL before this population was divided into four different MLTC.

Similar findings, pointing toward a different expression of TCR-specific sequences in distinct anatomical districts, have been obtained in rheumatoid arthritis (39). In fact, $V\beta14^+$ T cells have been found in higher frequency in synovial fluid of the affected joints than in PBL, and two cDNA clones characterized by particular $V\beta/D\beta/J\beta$ sequences accounted for 46–72% of all $V\beta14^+$ T cells, making up >4% of all T cells in the affected joints (39). In our analysis, between 18.4 and 19% of all $V\beta2^+$ sequences were associated with the tumor-specific TCR sequence, thus representing a proportion of similar magnitude (between 1.93 or 2.15%) of all TCR β chains present in the tumor infiltrate.

The finding that between 2,000 and 8,000 TCR β sequences had to be screened to detect evidence of selective overexpression of a melanoma-specific β chain may help to explain the lack of oligoclonality detected in other studies based on random sequencing of a smaller set of TCR transcripts obtained from TIL and control PHA blasts (10). In fact, our results indicate that in order to find evidence of selective expression of some TCR in the lymphoid infiltrate associated with a human tumor, information is needed on the sequence of a TCR shown to be linked to the recognition of the tumor. This information allowed us to upgrade to at least a factor 10, in comparison with previous reports (10), the magnitude of the pool of TCR-specific transcripts that could be analyzed.

The concept that specific TCR might be selectively expressed at the tumor site has been proposed by a previous study (9) showing that ocular melanomas from different patients are characterized by a predominant expression of $V\alpha7$, a V region gene segment different from the ones found expressed in the T cell clones of patient 9742. However, since the antigenic specificity of T cells expressing the $V\alpha7$ region was not assessed, it remains to be established whether T cells infiltrating ocular melanoma have similar function and anti-

genic specificity in comparison with CTL clones described in our study. A possibility to be investigated is that normal and neoplastic melanocytes associated with different tissues do not express the same set of genes. Therefore, melanomas from different tissues might express distinct antigenic profiles recognized by distinct sets of TCR. This hypothesis, if proven, may reconcile our results with those of Nitta et al. (9). However, the issue of which TCR are directed against melanoma-associated antigens is likely to be influenced not only by the antigenic profile of each tumor, but also by the MHC genotype of each patient. Only the comparison of specific antitumor T cells from different patients will clarify the relative contribution of antigens and HLA alleles to the shaping of the antitumor TCR repertoire.

Although the number of TCR sequences described here is too low to draw conclusions on the whole antimelanoma T cell response in patient 9742, the knowledge of TCR α and β chain sequences of human HLA-A2 restricted, melanoma-specific CTL that recognize shared melanoma Ags of broad or more selected distribution provides the molecular basis for studies aimed at defining whether other HLA-$A2^+$ melanoma patients show the same correlation between the fine specificity of antitumor T cell clones and the TCR gene elements they express. If so, the findings described in this study may have an impact on the development of improved therapeutic strategies for the adoptive immunotherapy of melanoma.

Finally, the association between the selective accumulation of a T cell clone at the tumor site and the peculiar antigenic specificity of this effector (namely recognition of Ags expressed not only on the neoplastic cells but also on normal melanocytes) raises the intriguing possibility that at least part of the host reaction to the tumor may be dependent on the recognition of tissue-related self-Ags. Clearly, as discussed in a recent report (15), we do not know whether the Ags recognized by our CTL clones on cultured melanocytes are expressed in vivo by this cell type. However, a possibility to be investigated is that the selective accumulation at the tumor site of a CTL clone expressing the $V\beta2.1/D\beta1/J\beta1.1/C\beta$ TCR reflects a process that has altered at some step the peripheral tolerance to tissue-related self-Ags (40).

The understanding of the mechanism that underlies both the accumulation/expansion at the tumor site of a melanocyte-lineage–specific T cell clone and the concomitant failure of an effective block of tumor growth at the same site, represent the next key issue to be addressed for clarifying the reasons of a seemingly insufficient immune response to the metastatic lesion.

References

1. Wölfel, T., E. Klehmann, C. Müller, K.H. Schütt, K.-H. Meyer zum Büschenfelde, and A. Knuth. 1989. Lysis of human melanoma cells by autologous cytolytic T cell clones: identification of human histocompatibility leukocyte antigen A2 as a restriction element for three different antigens. *J. Exp. Med.* 170:797.
2. Crowley, N.J., T.L. Darrow, M.A. Quinn-Aleen, and H.F. Seigler. 1991. MHC-restricted recognition of autologous melanoma by tumor-specific cytotoxic T cells. Evidence for restriction by a dominant HLA-A allele. *J. Immunol.* 146:1692.
3. Hom, S.S., S.L. Topalian, T. Simonis, M. Mancini, and S.A. Rosenberg. 1991. Common expression of melanoma tumor-associated antigens recognized by human tumor infiltrating lymphocytes: analysis by human lymphocyte antigen restriction. *J. Immunother.* 10:153.
4. Kawakami, Y., R. Zakut, S.L. Topalian, H. Stötter, and S.A. Rosenberg. 1992. Shared human melanoma antigens. Recognition by tumor-infiltrating lymphocytes in HLA-A2.1 transfected melanomas. *J. Immunol.* 148:638.
5. Traversari, C., P. Van Der Bruggen, I.F. Luescher, C. Lurquin, P. Chomez, A. Van Pel, E. De Plaen, A. Amar-Costesec, and T. Boon. 1992. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J. Exp. Med.* 176:1453.
6. Van den Eynde, B., P. Hainaut, M. Herin, A. Knuth, C. Lemoine, P. Weynants, P. van der Bruggen, R. Fauchet, and T. Boon. 1989. Presence on a human melanoma of multiple antigens recognized by autologous CTL. *Int. J. Cancer.* 44:634.
7. Anichini, A., A. Mazzocchi, G. Fossati, and G. Parmiani. 1989. Cytotoxic T lymphocyte clones from peripheral blood and from tumor site detect intratumor heterogeneity of melanoma cells. Analysis of specificity and mechanisms of interaction. *J. Immunol.* 142:3692.
8. Sensi, M.L., C. Castelli, A. Anichini, D. Grossberger, A. Mazzocchi, R. Mortarini, and G. Parmiani. 1991. Two autologous melanoma-specific and MHC-restricted human T cell clones with identical intra-tumor reactivity do not share the same TCR Vα and Vβ gene families. *Melanoma Res.* 1:261.
9. Nitta, T., J.R. Oksenberg, N.A. Rao, and L. Steinman. 1990. Predominant expression of T-cell receptor Vα7 in tumor-infiltrating lymphocytes of uveal melanoma. *Science (Wash. DC).* 249:672.
10. Ferradini, L., S. Roman-Roman, J. Azocar, M.-F. Avril, S. Viel, F. Triebel, and T. Hercend. 1992. Analysis of T-cell receptor α/β variability in lymphocytes infiltrating a melanoma metastasis. *Cancer Res.* 52:4649.
11. Albertini, M.R., J.A. Nicklas, B.F. Chastenay, T.C. Hunter, R.J. Albertini, S.S. Clark, J.A. Hank, and P.M. Sondel. 1991. Analysis of the T cell receptor β and γ genes from peripheral blood, regional lymph nodes and tumor infiltrating lymphocyte clones from melanoma patients. *Cancer Immunol. Immunother.* 32:325.
12. Belldegrun, A., A. Kasid, M. Uppenkamp, S.L. Topalian, and S.A. Rosenberg. 1989. Human tumor infiltrating lymphocytes. Analysis of lymphokine mRNA expression and relevance to cancer immunotherapy. *J. Immunol.* 142:4520.
13. Bennett, W.T., F. Pandolfi, B.H. Grove, G.E. Hawes, L.A. Boyle, R.L. Kradin, and J.T. Kurnick. 1992. Dominant rearrangement among tumor-infiltrating lymphocytes. Analysis of T-cells derived from 32 patients with melanoma, lung, and renal cell carcinoma. *Cancer (Phil.).* 69:2380.
14. Morita, T., M.A. Salmeron, R.P. Moser, M.I. Ross, and K. Itoh. 1992. Oligoclonal expansion of Vβ8+ cells in interleukin-2 activated T cells residing in subcutaneous metastatic melanoma. *Clin. & Exp. Metastasis.* 10:69.
15. Anichini, A., C. Maccalli, R. Mortarini, S. Salvi, A. Mazzocchi, P. Squarcina, M. Herlyn, and G. Parmiani. 1993. Melanoma cells and normal melanocytes share antigens recognized by HLA-A2-restricted cytotoxic T cell clones from melanoma patients. *J. Exp. Med.* 177:989.
16. Taswell, C., H.R. Mac Donald, and J.C. Cerottini. 1980. Clonal analysis of cytolytic T lymphocyte specificity. I. Phenotypically distinct sets of clones as the cellular basis of crossreactivity to alloantigens. *J. Exp. Med.* 151:1372.
17. Mortarini, R., A. Anichini, and G. Parmiani. 1991. Heterogeneity for integrins expression and cytokine-mediated VLA modulation can influence the adhesion of human melanoma to extracellular matrix proteins. *Int. J. Cancer.* 47:551.
18. Russo, C., A.K. Ng, M.A. Pellegrino, and S. Ferrone. 1983. The monoclonal antibody CR11.351 discriminates HLA-A2 variants identified by T cells. *Immunogenetics.* 18:23.
19. Accolla, R.S., N. Gross, S. Carrel, and G. Corte. 1981. Distinct forms of both α and β subunits are present in human Ia molecular pool. *Proc. Natl. Acad. Sci. USA.* 78:4549.
20. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.
21. Choi, Y., B. Kotzin, L. Herron, J. Callahan, P. Marrack, and J. Kappler. 1989. Interaction of *Staphylococcus aureus* toxin "superantigens" with human T cells. *Proc. Natl. Acad. Sci. USA.* 86:8941.
22. Uematsu, Y. 1991. A novel and rapid cloning method for the T-cell receptor variable region sequences. *Immunogenetics.* 34:174.
23. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, 2nd ed. Cold Spring Harbor, NY.
24. Uematsu, Y., H. Wege, A. Straus, M. Oh, W. Bannwarth, J. Lanchbury, G. Panayi, and M. Steinmetz. 1991. The T-cell receptor repertoire in the synovial fluid of a patient with rheumatoid arthritis is polyclonal. *Proc. Natl. Acad. Sci. USA.* 88:8534.
25. Wilson, R.K., E. Lai, P. Concannon, R.K. Barth, and L.E. Hood. 1988. Structure, organization and polymorphism of murine and human T-cell receptor α and β chain gene families. *Immunol. Rev.* 101:149.
26. Klein, M.H., P. Concannon, M. Everett, L.D.H. Kim, T. Hunkapiller, and L. Hood. 1987. Diversity and structure of

human T-cell receptor α chain variable region genes. *Proc. Natl. Acad. Sci. USA.* 84:6884.

27. Yoshikai, Y., N. Kimura, B. Toyonaga, and T.W. Mak. 1986. Sequences and repertoire of human T cell receptor α chain variable region genes in mature T lymphocytes. *J. Exp. Med.* 164:90.

28. Kimura, N., B. Toyonaga, Y. Yoshikai, R.-P. Du, and T.W. Mak. 1987. Sequences and repertoire of the human T cell receptor α and β chain variable region genes in thymocytes. *Eur. J. Immunol.* 17:375.

29. Roman-Roman, S., L. Ferradini, J. Azocar, C. Genevée, T. Hercend, and F. Triebel. 1991. Studies on the human T-cell receptor α/β variable region genes. I. Identification of 7 additional Vα subfamilies and 14 Jα gene segments. *Eur. J. Immunol.* 21:927.

30. Concannon, P., L. Pickering, P. Kung, and L. Hood. 1986. Diversity and structure of human T-cell receptor β chain variable region genes. *Proc. Natl. Acad. Sci. USA.* 83:6598.

31. Kimura, N., B. Toyonaga, Y. Hoshikai, F. Triebel, P. Debre, M.D. Minden, and T.W. Mak. 1986. Sequences and diversity of human T cell receptor β chain variable region genes. *J. Exp. Med.* 164:739.

32. Plaza, A., D.H. Kono, and A.N. Theofilopoulos. 1991. New human Vβ genes and polymorphic variants. *J. Immunol.* 147:4360.

33. Ferradini, L., S. Roman-Roman, J. Azocar, H. Michalaki, F. Triebel, and T. Hercend. 1991. Studies on the human α/β T-cell receptor variable region genes. II. Identification of four

34. Toyonaga, B., Y. Yoshikai, V. Vadasz, B. Chin, and T.W. Mak. 1985. Organization and sequences of the diversity, joining, and constant region genes of the human T-cell receptor β chain. *Proc. Natl. Acad. Sci. USA.* 82:8624.

35. Sensi, M.L., C. Castelli, S. Salvi, A. Mazzocchi, R. Mortarini, G. Nicolini, A. Anichini, and G. Parmiani. 1992. Expansion of major histocompatibility complex-restricted anti-melanoma cytotoxic T-cell lymphocyte clones with identical T-cell receptor from tumor-infiltrating lymphocytes. *J. Immunother.* 12:207.

36. Chothia, C., D.R. Boswell, and M.A. Lesk. 1988. The outline structure of the T-cell αβ receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3745.

37. Parmiani, G., A. Anichini, and G. Fossati. 1990. Cellular immune response against autologous human melanoma: are in vitro studies providing a framework for a more effective immunotherapy? *J. Natl. Cancer Inst.* 82:361.

38. Coulie, P.G., M. Somville, F. Lehmann, P. Hainaut, F. Brasseru, R. Devos, and T. Boon. 1992. Precursor frequency analysis of human cytolytic T lymphocytes directed against autologous melanoma cells. *Int. J. Cancer.* 50:289.

39. Paliard, X., S.G. West, J.A. Lafferty, J.R. Clements, J.W. Kappler, P. Marrack, and B.L. Kotzin. 1991. Evidence for the effects of a superantigen in rheumatoid arthritis. *Science (Wash. DC).* 253:325.

40. Arnold, B., G. Schronrich, and G.J. Hammerling. 1993. Multiple levels of peripheral tolerance. *Immunol. Today.* 14:12.